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ON THE ABSENCE OF CIRCADIAN RHYTHMICITY IN *DROSOPHILA PSEUDOOBSCURA* PUPAE

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Circadian rhythms are the rule rather than the exception in eucaryotic organisms; from unicellular organisms to higher plants and animals a diversity of physiological functions has been shown to exhibit approximately 24 hour intervals between maxima. In spite of this physiological and systematic ubiquity, the *circadian oscillations* underlying circadian rhythms are remarkably uniform in their formal properties: they persist in constant dark and different constant temperatures with a period, τ, which is close to 24 hours, and they can be reset, and entrained by light and temperature signals (for reviews, see Pittendrigh, 1960; Bunning, 1964; and Aschoff, 1965). Furthermore, circadian oscillations have been exploited by organisms for a variety of uses, such as compensation for sun movement in navigation, synchronization of social behavior and measurement of photoperiod; some of these uses are surely unrelated to the original sources of selection for circadian oscillations (Hoffman, 1960; Pittendrigh, 1961, 1966). These findings have led some authors to propose that circadian oscillations are an ancient and integral part of eucaryote physiology (Pittendrigh, 1960, 1961, 1966; Halberg, 1960; and Bunning, 1964), and two authors have suggested that circadian oscillations inhere in the organization and reading of the genetic message (Ehret and Trucco, 1967).

On the other hand, it has long been known that many plants and animals which normally manifest circadian rhythms do not manifest them under certain environmental conditions. These types of "arhythmicity" may be divided into two broad categories corresponding to the sequence of experimental conditions which cause it: (1) Primary arhythmicity: Circadian rhythms are not evident and they do not develop if an organism or population of organisms is raised (from seed or egg) in constant temperature and constant light or dark. (2) Secondary arhythmicity: Once induced by a periodic environment (light and/or temperature signals), circadian rhythms can be inhibited by (a) constant very low temperature, (b) an oxygen-depleted atmosphere, or, (c) constant bright light (Bunning, 1964; Wilkins, 1965). In primary arhythmic organisms, a circadian rhythm can be induced by single or repeated signals (light or temperature). And in secondary arhythmic organisms, the circadian rhythm can be restored by return to non-inhibitory conditions (higher temperature, oxygenated atmosphere, dim light).

As several authors have noted (Pittendrigh and Bruce, 1957; Wasserman, 1959; Sweeney and Hastings, 1960), these two types of arhythmicity could be interpreted as due to *asynchrony* or *arhythmicity* of constituent parts (organelles in cells; cells or organs in individuals; individuals in populations); that is, either the constituent parts are not oscillating (arhythmicity), or the constituent parts are oscillating, but with their phases distributed randomly (asynchrony). This paper discusses some old and new facts bearing on these two possible interpretations

and the meaning of these facts for any hypothesis to the effect that circadian rhythmicity is an essential component of physiological organization.

First, concerning primary arhythmicity, an experimental distinction between the two interpretations is made possible by the finding that a single light or temperature signal induces a circadian rhythmicity in primary arhythmic individuals and populations (Pittendrigh, 1954; Wilkins, 1965). The question may be restated in terms of this phenomenon: "Is the *induction* of circadian rhythmicity in primary arhythmic populations due to *initiation* of circadian oscillations inherited at rest, or to *synchronization* of circadian oscillations inherited in motion but out of phase?" As Pittendrigh and Bruce (1957) noted, this question may be answered by comparing the effects of a light or temperature signal (a) when it acts to phase shift (reset) a population of circadian oscillations known to be running and synchronized, and (b) when it acts to induce rhythmicity in a primary arhythmic population.

Pittendrigh (1954) found that either a 4 hour light pulse or a 4 hour temperature pulse (16°/26°/16° C) induces a circadian rhythm in adult emergence in primary arhythmic populations of *Drosophila pseudoobscura* pupae.

Later, Pittendrigh and Bruce (1957) compared the inducing and phase shifting effects of light signals on the *Drosophila* rhythm, and found the results in support of the synchronization hypothesis. However, their experiments showed only the possible correctness of the synchronization hypothesis, because the light signals they used can generate up to 12 hour phase shifts; the possibility of rhythm *initiation* by the light signals was not excluded. In fact, concerning rhythm induction by a 4 hour temperature pulse (16°/26°/16° C), Sweeney and Hastings (1960) pointed out that if this temperature pulse generates only small phase shifts when applied to the free running oscillation, then it *a priori* could not synchronize a population of running but randomly phased, 24 hour oscillations. They inferred that the 4 hour temperature pulse generates small phase shifts on the basis of the finding that a temperature step (26°/16° C) generates only small phase shifts when applied to the free running oscillation (Pittendrigh, Bruce and Kaus, 1958).

In this paper a comparison is made between the phase-shifting and inducing effect of the same temperature signal on the emergence rhythm in *Drosophila pseudoobscura* pupae.

Materials and Methods

Two types of automatic collection devices have been developed for routine assay of the *Drosophila* emergence rhythm. The first type of collection device was used in the recently reported phase shift experiments (Zimmerman, Pittendrigh and Pavlidis, 1968); its use involves rearing pupae in plastic boxes, collecting them by flotation, and gluing them to a brass plate. For the rhythm induction experiments reported here, a second type of collection device was designed in which the flies undergo their complete life cycle; the collection of pupae by flotation in the light is thus avoided. Each such device consists of 4 hollow-walled lucite cups mounted on a threaded steel rod, and surrounded by a cannister made of 4-inch diameter lucite tubing and a plastic funnel. Parent flies lay eggs on food inside the cups, and later the larvae crawl out and pupate on rug yarn wrapped around the walls of the cup. The cannister surrounding the cups is suspended from a solenoid whose periodic actuation lifts and drops the system, thus shaking flies

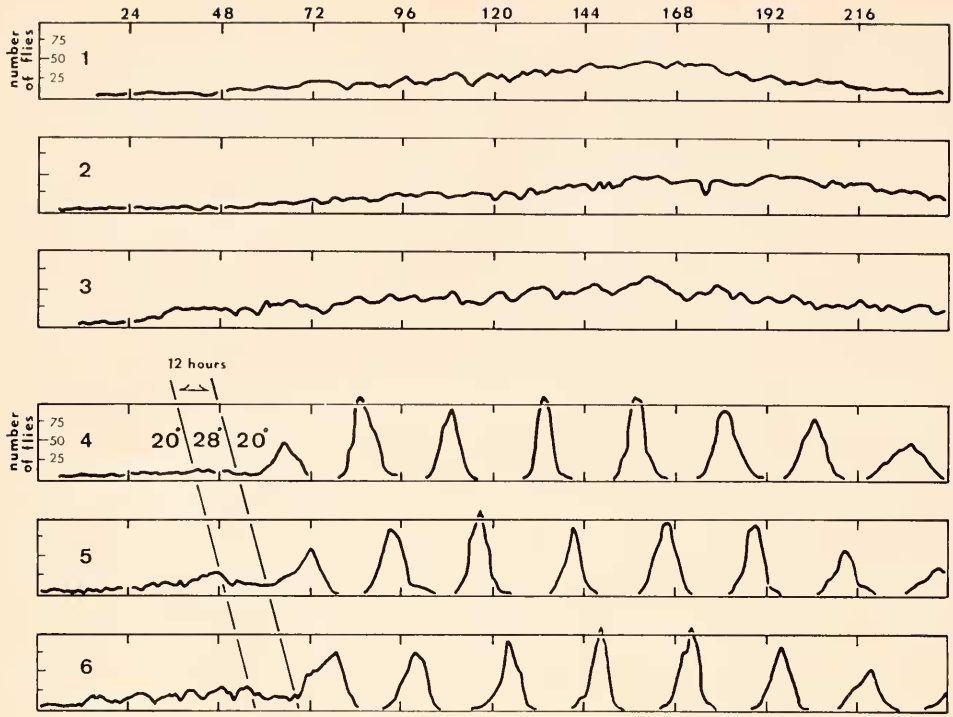

FIGURE 1. Arhythmicity and the induction of a circadian rhythmicity in populations of *Drosophila pseudoobscura*. Shown are the number of adult flies which emerged per hour over a 10 day period from 6 populations of pupae of mixed developmental ages. The top 3 populations were kept throughout in constant dark and at constant 20° C. The bottom 3 populations were kept in constant dark, but were exposed to a single 12 hour high temperature pulse (12 hours at 28° C); the pulses were started at successive 8 hour intervals (see text).

which have emerged into a vial of detergent solution into which the cannister vents (for details, see Zimmerman, 1966). Temperature control is achieved by pumping water through the hollow-walled lucite cups; each set of cups is coupled via pumps and water valves to two water baths—one at constant 20° C and the other at constant 28° C. Automatic time switches are set to turn on and off the valves and pumps, thus switching the water flow through the cups from one temperature-controlled bath to another. Control of the light regime is provided by 4 watt white fluorescent bulbs connected to time switches. Unlike the collection devices used in the phase shift experiments—which involved collection of pupae by flotation in the light—these new collection devices guarantee constant conditions throughout the organism's life cycle.

RESULTS

Arhythmicity and the induction of rhythmicity

Figure 1 shows the number of flies which emerged per hour for 10 days in 6 populations of *Drosophila pseudoobscura*. The upper 3 populations were kept in

constant dark (DD) and constant 20° C throughout (egg to adult); the lower 3 populations were exposed to a single 12 hour high temperature pulse (20°/28°/20° C) after emergence had begun. It is clear that the 3 populations kept throughout in constant conditions are arhythmic: emergence occurs randomly throughout the day. The lower 3 populations were also *initially aperiodic,* but after exposure to the temperature pulse a circadian rhythmicity in adult emergence is induced. Furthermore, the *phase* of the induced emergence rhythm is determined by the time (local) when the pulse was given.

Phase shifting the rhythm by a temperature pulse

In the upper part of Figure 2 is shown the rhythm phase shifting effect of the 12 hour high temperature pulse (20°/28°/20° C): 12 populations of pupae were raised in an LD 12:12 cycle (12 hours light/12 hours dark) at constant 20° C, placed in DD (after the "final dusk"), and exposed to the temperature pulse at successively later times (2 hour intervals). The plotted points are the median hours of the emergence peaks for each day. The dotted vertical lines show the daily median emergence hour of the "free run control"—a population of pupae released into DD and constant 20° C, but *not* subjected to the temperature pulse.

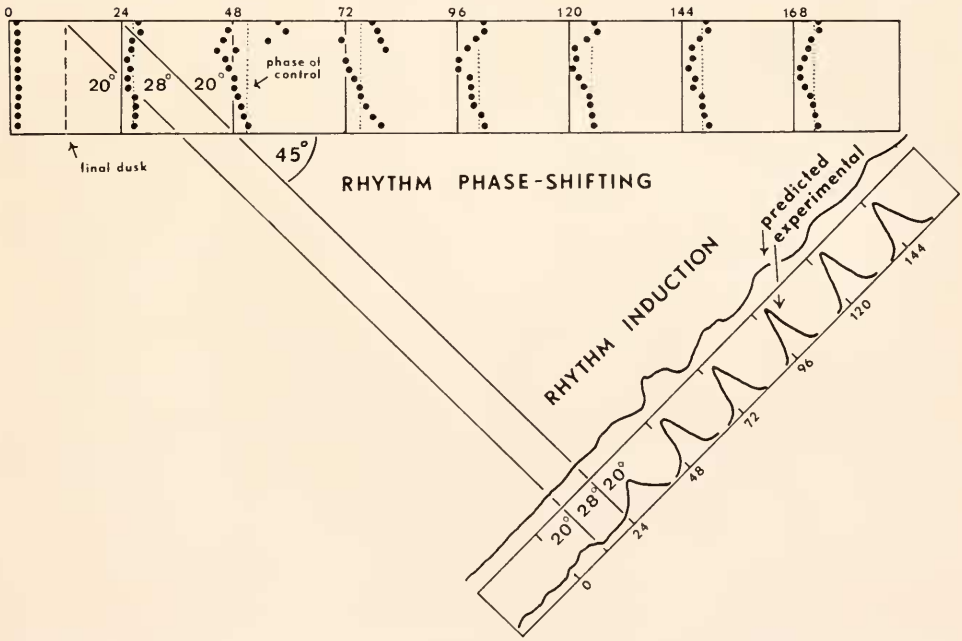

FIGURE 2. Comparison of the rhythm-phase shifting and rhythm-inducing effects of a 12 hour high temperature pulse (20°/28°20° C). In the upper part of the figure are shown the daily median emergence hours of 12 populations of *Drosophila pseudoobscura* exposed to the temperature pulse at successively later times (2 hour intervals). Forty-five degree summation of the number of flies which emerged per hour from these 12 populations yields the "predicted" emergence distribution plotted in the lower part of the figure. The "experimental" distribution was obtained by pooling the lower 3 emergence distributions in Figure 1 (after normalizing these distributions to the onset of the temperature pulse). See text.

Large transient phase shifts are evident on the first and second days after the pulse. However, the final steady state phase shifts—evident on the fifth and sixth days after the pulse—are small; the maximum delay phase shift is 1.3 hours, and the maximum advance phase shift is 3.3 hours. This experiment thus characterizes the magnitude and direction of the phase shift generated by the temperature pulse as a function of the point in the circadian oscillation's cycle exposed to the signal (Zimmerman, Pittendrigh and Pavlidis, 1968; *cf.* Pittendrigh and Minis, 1964).

Discussion

The rhythm phase shifting and rhythm induction effects of the temperature pulse are compared in Figure 2. Forty-five degree projection of the phase shifting data *before the pulse* synthesizes (in the lower part of Figure 2) a model population of pupae which is arhythmic, but which is known to consist of individuals whose oscillations are in motion with phases distributed randomly. Forty-five degree projection of the phase shift data *during and after the pulse* thus simulates the synchronization hypothesis by subjecting the model population of running but asynchronous oscillations to a synchronizing (phase shifting) signal. The prediction resulting from this 45 degree summation of the phase shifting experiment illustrates what is *a priori* clear: a signal which generates steady state phase shifts of only a few hours is incapable of synchronizing a population of running but asynchronous circadian (~24 hour) oscillations.

Below the synthetic distribution—"predicted" from the synchronization hypothesis—is the "experimental" emergence distribution of an arhythmic population of pupae exposed to the temperature pulse; these data are pooled from the lower three experiments shown in Figure 1. The results were previously clear: the temperature pulse does induce a circadian rhythmicity in emergence. The synchronization hypothesis is thus excluded, and we can conclude that the circadian oscillation in individual flies is inherited at rest, and that it is *initiated* (set in motion) by the first light or temperature signal.

Turning now to secondary arhythmicity, the possible interpretations may be formulated in a question analogous to that posed for primary arhythmicity: Is the loss of overt rhythmicity in individuals and populations due to a *damping out* of circadian oscillations in constituent parts, or to a *desynchronization* of circadian oscillations which continue to run in constituent parts? The best experiments bearing on this question are those of Sweeney (1960) on the marine dinoflagellate, *Gonyaulax*. She found that both individual cells and populations of cells show a circadian rhythm if placed in constant dim light (50 footcandles) after a previous LD cycle; however, the rhythm is lost in both individual cells and populations of cells if they are placed in constant bright light (800 footcandles) after a previous LD cycle. Wasserman (1959) showed that the circadian rhythm of leaf movement in the plant *Phaseolus* is accompanied by a parallel circadian rhythm in nuclear volume of epidermal cells; if plants are placed in constant bright light after an LD cycle, both rhythmicities cease. Thus, in these two cases, secondary arhythmicity may be attributed to an arhythmicity of constituent parts.

On the other hand, primary and secondary arhythmicity have been discussed in some cases as due, respectively, to asynchrony and desynchronization of oscillatory constituent parts; but discussion of the evidence is beyond the intended scope

of this paper (see Bunning, 1964). My point here is first to emphasize that in several well-studied experimental systems (*Drosophila* emergence and others cited) the absence of overt circadian rhythmicity may be attributed to a true arhythmicity of constituent parts; and second, that this finding, considered in conjunction with the fact that most organisms can reproduce and function normally in aperiodic environments, presents definite difficulties for (a) the general notion that the maintenance and entrainment of circadian oscillations is essential to the normal physiology and development of eucaryotic organisms (Pittendrigh, 1960, 1961; Bunning, 1964), and (b) the more specific hypothesis of Ehret and Trucco (1967) that the mechanism for circadian oscillations inheres in the physical organization—and therefore transcription—of the DNA in eucaryotic cells.

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Summary

1. Although circadian rhythms are systematically and physiologically ubiquitous in eucaryotic organisms, they are not evident under certain experimental conditions. For example, there is no circadian rhythm in adult emergence in populations of *Drosophila pseudoobscura* if the organism is raised in constant dark and temperature.

2. In general, such overt arhythmicity could be interpreted as due to asynchrony or true arhythmicity of constituent parts (organelles in cells; cells and organs in individuals; individuals in populations).

3. In the case of the circadian rhythm of adult emergence in *Drosophila pseudoobscura*, a distinction between these two interpretations of arhythmicity is made possible by comparing the rhythm-phase shifting and rhythm-inducing effect of the same temperature signal. It was concluded that arhythmicity of *Drosophila* populations was due to a true arhythmicity of constituent parts (individual flies).

4. Other experiments are mentioned in which the arhythmicity of populations and individuals is attributable to a true arhythmicity of constituent parts.

5. This finding presents difficulties for hypotheses asserting the importance of circadian rhythmicities in the physiology of eucaryotic organisms.

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